# Effects of Dietary Oils and Methyl Ethyl Ketone Peroxide on *in vivo* Lipid Peroxidation and Antioxidants in Rat Heart and Liver

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## Abstract:

Weanling male Sprague-Dawley rats were fed diets for four weeks which differed in their content of n-6 (corn oil; CO) and n-3 fatty acids (fish oil; FO), but were similar in their content of saturated and monounsaturated fatty acids and vitamin E. At the end of the four-week feeding period, each dietary group was subdivided into two groups. One group received a single placebo injection of α-tocopherol-stripped corn oil (TSCO); the other group received a single injection of the free radical generator, methyl ethyl ketone peroxide (MEKP), in TSCO. Twenty-four hours after injection, the effect of dietary oil and MEKP treatment on endogenous lipid peroxide (LPO) production (measured as methylene blue formed by the "Determiner LPO" assay), glutathione (GSH) and vitamin E content, and fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in heart and liver from unfasted animals were measured. FO-fed rats had significantly heavier hearts and livers, increased levels of n-3 fatty acids in membrane phospholipids, and higher liver LPO levels than CO-fed rats. MEKP treatment resulted in significantly lower body weights and liver GSH levels. The data indicate that dietary n-3 fatty acids increase lipid peroxidation in liver somewhat more than in heart. The study also demonstrates that the effect of induced oxidative stress due to a single dose of MEKP on lipid peroxide formation and antioxidant status in tissues from unfasted animals was independent of the dietary oils.

## Article:

Dietary supplementation with highly polyunsaturated fats, such as those containing the n-3 fatty acids found in fish oil, is associated with an increased tendency for lipid peroxide formation in certain tissues (1-3). Lipid peroxidation mediated by free radicals is considered to be a prevalent mechanism of cell membrane destruction and cell damage and has been suggested to be associated with the initiation and development of atherosclerosis (4). Little is known about how the organism responds to an increase in oxidative stress (i.e., activation of free-radical reactions), such as lipid peroxidation. In view of the suspected role of oxidative degradation of lipids in the etiology of atherogenesis, such information is useful.

Dietary fatty acids influence the composition of membrane phospholipids in heart and liver (5,6). Indeed, membrane phospholipids with a high level of polyunsaturated fatty acids (PUFA) are more sensitive to hydroxy radicals (7,8). Susceptibility to peroxidation is also influenced by free radical scavengers and antioxidants such as vitamin E and glutathione (GSH) (9). Induced oxidative stress is accompanied by a decrease in the content of the antioxidants vitamin E and/or GSH (10). Further, increased lipid peroxidation is associated with a decrease in liver GSH (11-13).

In the above experiments, animals were fasted overnight prior to sampling. The standard practice to fast animals overnight before drug administration in experimental research is to ensure uniform absorption of the test compound. However, deprivation of food and/or water is a powerful stressor. Experiments in rats have shown that food deprivation induces alterations in free radical-scavenging systems that differ from tissue to tissue (14). Elevated turnover rates of liver GSH have been reported during fasting in rats (15). Furthermore, fasting in rats lowers the GSH level of the liver without changing its level in the heart (15-17).

Data that indicate that feeding fish oil (FO) induces higher lipid peroxidation in tissues has been based on the thiobarbituric acid reactive substances (TBARS) test. This method measures the end product, malondialdehyde, which results from the breakdown of lipid peroxides (LPOs) and nonlipid compounds. Several investigators have criticized the use of the TBARS test since the test is not a reliable index of lipid peroxidation in organ systems (18-20). A preferable procedure is to measure LPO levels, using the Determiner LPO assay, a colorimetric method that specifically detects hydroperoxides, endoperoxides and peroxy radicals (20,21).

The present study was undertaken to investigate the effects of dietary n-6 and n-3 PUFA and free radicals generated by methyl ethyl ketone peroxide (MEKP) on the extent of lipid peroxidation and on antioxidant status, i.e., vitamin E and GSH content, in heart and liver tissues of rats. MEKP is a toxic, lipid-soluble peroxide that has been shown to rapidly initiate lipid peroxidation in vivo and in vitro (22,23). To avoid the possible potentiating effect of fasting on detoxification systems, the effect of MEKP on in vivo oxidation processes was studied in unfasted normal animals. The lipid peroxidation was measured by a new method, the Determiner LPO assay (20,21).

## MATERIALS AND METHODS

### *Animals and diets.*

Forty-one male weanling Sprague-Dawley rats were obtained from Simonson Laboratories (Los Angeles, CA). The rats were housed individually in wire cages under standard conditions of 22°C, 45% relative humidity, using a 12-h light-dark cycle. The animal care procedures used in this study were approved by the Animal Care and Use Committee at Oregon State heavier hearts and livers than those fed the CO-rich diet. These differences prevailed when the data were expressed on a relative weight basis. The livers of the MEKP-treated rats were lighter than those that did not receive the injection, when the data were expressed on a absolute basis but not on a relative basis.

The highly polyunsaturated n-6 and n-3 fatty acids in the diets were efficiently incorporated into the major classes of membrane phospholipids, phosphatidylcholine (PC) and

phosphatidylethanolamine (PE; data not shown), of heart and liver. The fatty acid levels of phospholipids in heart and liver were significantly different between the two dietary groups with respect to most of the fatty acids analyzed. Pronounced increases in n-3 fatty acids in heart and liver membrane phospholipids have been shown repeatedly, and since the effect of the treatment and the interaction between the effects of dietary oil and treatment caused minimal changes in the fatty acid levels of heart and liver PE, the data from PE are not given here. The interaction between dietary oil and treatment was significant in six fatty acids and in the PI of heart PC (Table 3) and in two fatty acids of liver PC (Table 4). Within the CO group, MEKP treatment significantly decreased the level of 18:2n-6. Within the FO group, the level of 16:0 was significantly increased, and the levels of 20:4n-6, 22:5n-3, 22:6n-3 and the PI were significantly decreased after a single MEKP injection. MEKP treatment changed the fatty acid levels of liver PC only within the CO group, where the levels of 18:1n-9 and 18:2n-6 were decreased. In heart PE, there was one interaction where the PI values decreased in MEKP-injected FO-fed rats from 239.2 ± 3.3% to 221.3 ± 4.3% but remained virtually unchanged in CO-fed rats (148.8 ± 2.0% to 146.6 ± 4.5%).

The effects of the dietary oils and MEKP treatment on GSH, vitamin E and LPO levels are shown in Table 5. Liver GSH was significantly reduced ($P < 0.003$) by MEKP treatment while dietary oils had no effect. Heart GSH was unaffected by dietary oil or MEKP treatment. Liver GSH was roughly three times that of the heart. Vitamin E in heart and liver was unaffected by dietary oil or MEKP treatment. However, heart vitamin E was roughly twice that in the liver (2 nmol/mg protein vs. 1, respectively). Liver LPO was significantly higher ($P < 0.05$) in the FO-fed group compared to the CO-fed group, while heart LPO was unaffected. MEKP treatment did not affect the LPO levels in heart or liver in either of the dietary groups.

Figure 2 shows the relationship between PI in heart PC, vitamin E and LPO levels from rats on the FO-diet and being MEKP-treated. The PI was positively correlated with vitamin E levels and negatively correlated with LPO levels.

## DISCUSSION

The present study has shown that rats fed n-3 enriched diets do not respond differently to induced oxidative stress from a single dose of MEKP as evidenced by LPO formation and antioxidant status, compared to rats fed n-6 enriched diets. As anticipated, the injection of MEKP stressed the animals, as evidenced by their weight loss within 24 h after injection (Fig. 1). The changes in body weight effected by MEKP might be due to loss of appetite and lower food intake in addition to metabolic changes. The injection of TSCO or the handling associated with the injection was also a stressor, as evidenced by the smaller weight gain experienced by the rats after this injection.

**TABLE 3**

**Effect of Dietary Oil and MEKP Treatment on the Fatty Acid Composition of Heart Phosphatidylcholine[a]**

| | Corn oil | | Fish oil | | *P*-value[b] | | |
|---|---|---|---|---|---|---|---|
| | TSCO | MEKP | TSCO | MEKP | O | T | O × T |
| Fatty acids | | | | | | | |
| 14:0 | 0.11 ± 0.11 | 0.06 ± 0.06 | 0.22 ± 0.14 | 0.41 ± 0.32 | NS | NS | NS |
| 16:0 | 14.64 ± 0.52 | 13.65 ± 0.50 | 16.36 ± 0.33 | 20.29 ± 2.12[d] | 0.002 | NS | 0.05 |
| 16:1n-7 | | | | | | | |
| 18:0 | 30.26 ± 0.44 | 31.73 ± 0.86 | 27.42 ± 0.45 | 28.21 ± 2.45 | 0.03 | NS | NS |
| 18:1n-9 | 4.43 ± 0.16 | 3.94 ± 0.10 | 2.64 ± 0.11 | 3.14 ± 0.26 | <0.0001 | NS | 0.01 |
| 18:1n-7 | 2.51 ± 0.17 | 2.42 ± 0.08 | 3.02 ± 0.14 | 3.47 ± 0.51 | 0.01 | NS | NS |
| 18:2n-6 | 13.24 ± 0.59 | 9.87 ± 1.01[e] | 2.64 ± 0.13 | 2.62 ± 0.25 | <0.0001 | 0.01 | 0.01 |
| 20:4n-6 | 28.04 ± 1.05 | 30.08 ± 1.30 | 21.35 ± 0.34 | 17.81 ± 1.44[d] | <0.0001 | NS | 0.02 |
| 20:5n-3 | | | 4.16 ± 0.25 | 3.43 ± 0.20 | | 0.02 | |
| 22:4n-6 | 1.52 ± 0.21 | 0.87 ± 0.55 | | | | NS | |
| 22:5n-3 | 1.04 ± 0.10 | 1.37 ± 0.39 | 3.65 ± 0.13 | 2.87 ± 0.28[d] | 0.0001 | NS | 0.04 |
| 22:6n-3 | 2.31 ± 0.15 | 3.28 ± 0.60 | 17.48 ± 0.20 | 13.66 ± 1.68[e] | 0.0001 | NS | 0.02 |
| n-6/n-3 | 12.83 ± 1.19 | 10.23 ± 1.79 | 0.95 ± 0.01 | 1.05 ± 0.11 | 0.0001 | NS | NS |
| PI[c] | 118.21 ± 2.93 | 124.75 ± 6.73 | 184.88 ± 2.09 | 149.58 ± 13.31[e] | 0.0001 | NS | 0.02 |

[a]Data are expressed as relative weight percent of total fatty acids. Values are means ± SEM for five rats. See Table 2 for other abbreviations.

[b]NS, not significant at $P < 0.05$, analysis of variance.

[c]PI, peroxidizability index, (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

[d]$P < 0.05$, [e]$P < 0.01$, compared to TSCO-treated rats, Fischer's least significant difference.

**TABLE 4**

**Effect of Dietary Oil and MEKP Treatment on the Fatty Acid Composition of Liver Phosphatidylcholine[a]**

| | Corn oil | | Fish oil | | *P*-value[b] | | |
|---|---|---|---|---|---|---|---|
| | TSCO | MEKP | TSCO | MEKP | O | T | O × T |
| Fatty acids | | | | | | | |
| 14:0 | 0.35 ± 0.09 | 0.30 ± 0.08 | 0.86 ± 0.08 | 0.85 ± 0.11 | 0.0001 | NS | NS |
| 16:0 | 19.33 ± 0.60 | 21.13 ± 1.24 | 28.26 ± 0.88 | 30.08 ± 2.05 | 0.0001 | NS | NS |
| 16:1n-7 | | | 2.24 ± 0.16 | 1.70 ± 0.45 | | NS | |
| 18:0 | 22.04 ± 0.60 | 24.10 ± 0.37 | 14.08 ± 0.30 | 14.54 ± 0.40 | 0.0001 | 0.010 | NS |
| 18:1n-9 | 4.73 ± 0.07 | 3.65 ± 0.22[d] | 6.14 ± 0.28 | 6.40 ± 0.44 | 0.0001 | NS | 0.03 |
| 18:1n-7 | 1.33 ± 0.14 | 1.18 ± 0.09 | 2.60 ± 0.15 | 2.73 ± 0.16 | 0.0001 | NS | NS |
| 18:2n-6 | 16.14 ± 0.29 | 13.99 ± 0.30[e] | 7.95 ± 0.22 | 8.51 ± 0.41 | 0.0001 | 0.02 | 0.001 |
| 20:4n-6 | 27.70 ± 0.46 | 27.71 ± 0.64 | 11.25 ± 0.98 | 9.93 ± 0.60 | 0.0001 | NS | NS |
| 20:5n-3 | | | 10.29 ± 1.17 | 9.08 ± 1.06 | | NS | |
| 22:4n-6 | 0.65 ± 0.18 | 0.54 ± 0.22 | | | | NS | |
| 22:5n-3 | 0.46 ± 0.23 | 0.67 ± 0.07 | 1.93 ± 0.18 | 1.70 ± 0.20 | 0.0001 | NS | NS |
| 22:6n-3 | 3.17 ± 0.19 | 3.61 ± 0.26 | 11.98 ± 0.45 | 10.87 ± 1.27 | 0.0001 | NS | NS |
| n-6/n-3 | 12.92 ± 0.94 | 10.26 ± 0.81 | 0.87 ± 0.11 | 0.91 ± 0.08 | 0.0001 | NS | NS |
| PI[c] | 121.19 ± 1.13 | 120.38 ± 2.46 | 152.60 ± 4.07 | 136.99 ± 12.03 | 0.002 | NS | NS |

[a]Data are expressed as relative weight percent of total fatty acids. Values are means ± SEM for five rats. See Table 2 for other abbreviations.

[b]NS, not significant at $P < 0.05$, analysis of variance.

[c]PI, peroxidizability index, (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

[d]$P < 0.05$, [e]$P < 0.001$, compared to TSCO-treated rats, Fischer's least significant difference.

This occurred despite the fact that the rats were handled frequently and that great care was taken to assure that the injection was made causing a minimum of discomfort. The data suggest that stress can confound results from studies in which animals are subjected to procedures such as an injection.

The highly polyunsaturated n-6 fatty acids in CO and n-3 catty acids in FO were efficiently incorporated into the major classes of phospholipids, i.e., PC and PE, of heart and liver, which is in agreement with previous studies (5,6). The contribution of individual fatty acids to lipid peroxidation has been shown to reflect the degree of un saturation of n-3 fatty acids more than that of the n-6 type (31). In our study, a single MEKP injection caused significant reduction in the levels of PUFA, 20:4n-6, 22:5n-3 and 22:6n-3 of heart PC from FO-fed rats. The interaction between dietary FO and MEKP treatment on fatty acid levels was evident to a lesser extent in heart PE (data not shown) and liver PC. The fatty acid levels of liver PE (data not shown) were unaffected by MEKP in each of the dietary groups. In sarcolemma of myocardial cells, PC is preferentially present in the outer monolayer, and PE in the inner (32). The observed reduction in the levels of highly PUFA in heart PC could indicate that PC is less

**TABLE 5**

**Effect of Dietary Oil and MEKP Treatment on Glutathione (GSH), Vitamin E and Lipid Peroxide (LPO) Content of Heart and Liver[a]**

| | Corn oil | | Fish oil | | *P*-value[b] | | |
|---|---|---|---|---|---|---|---|
| | TSCO | MEKP | TSCO | MEKP | O | T | O × T |
| Heart | | | | | | | |
| GSH | 9.6 ± 0.5 | 8.9 ± 0.8 | 9.2 ± 0.6 | 9.4 ± 0.6 | NS | NS | NS |
| Vitamin E | 2.09 ± 0.10 | 2.03 ± 0.14 | 1.85 ± 0.11 | 2.17 ± 0.15 | NS | NS | NS |
| LPO | 0.040 ± 0.006 | 0.029 ± 0.004 | 0.033 ± 0.007 | 0.024 ± 0.005 | NS | NS | NS |
| Liver | | | | | | | |
| GSH | 31.2 ± 2.1 | 19.8 ± 2.6 | 28.0 ± 2.1 | 24.5 ± 2.5 | NS | 0.003 | NS |
| Vitamin E | 1.15 ± 0.13 | 0.90 ± 0.12 | 0.90 ± 0.04 | 0.88 ± 0.07 | NS | NS | NS |
| LPO | 0.053 ± 0.008 | 0.043 ± 0.007 | 0.066 ± 0.10 | 0.074 ± 0.014 | 0.05 | NS | NS |

[a]Values are reported as nmol/mg protein and are means ± SEM for 10–11 rats. See Table 2 for other abbreviations.

[b]NS, not significant at $P < 0.05$.

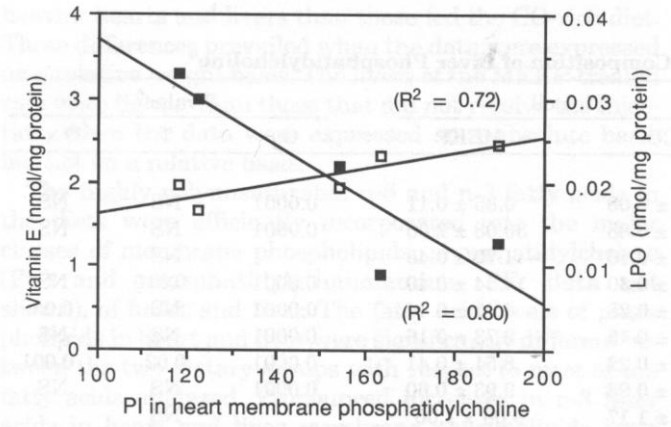


**FIG. 2. Relationship between peroxidizability index (PI) in phosphatidylcholine, vitamin E (open symbols) and lipid peroxide (LPO, closed symbols) levels in heart from rats on fish oil-rich diet being methyl ethyl ketone peroxide treated.**

protected from oxidation induced by MEKP. The observed changes in fatty acid composition of heart phospholipids did not resemble changes induced by stress, i.e., exogenous epinephrine (5). Repeated administration of epinephrine caused increased levels of 20:4n-6 and 22:6n-3 in PC, decreased or unaltered 20:4n-6 and increased 22:6n-3 in PE and decreased level of 18:2n-6 in PC and PE. These changes were qualitatively similar although different dietary fats were used, i.e., butter, corn oil or cod liver oil (5).

The phospholipids of heart and liver from the FO-fed rats contain high levels of the highly peroxidizable polyunsaturated n-3 fatty acids (Tables 3 and 4) causing the higher PI values. Even though the PI of the phospholipids was higher in heart than in liver of FO-fed rats, lipid peroxidation was higher in the liver, i.e., the LPO levels were higher in liver than in heart of FO-fed rats (Table 5). Vitamin E is thought to act as one of the most important antioxidants in vivo because of its role in blocking the propagation of LPO in the membrane. Vitamin E levels in the heart were twice those in the liver, and LPO levels were lower in heart than in liver. The insensitivity of heart from FO-fed rats to lipid peroxidation could be explained by the high vitamin E level, which seems to be sufficient to suppress the in vivo peroxidation of n-3 fatty acids in heart phospholipids of normal rats.

Mouri et al. (3) and Chautan et al. (33) showed that the heart vitamin E content dramatically increased as the membranes became enriched in n-3 PUFA, whereas liver became depleted of vitamin E. However, in our study, no differences in the level of vitamin E in heart or liver were found between FO-fed and CO-fed rats (Table 5). The role of vitamin E in preventing the oxidative destruction of PUFA in cell membranes has been elusive (34). It has been suggested that vitamin E stabilizes the cell membrane through interaction of its phytyl side chains with the polyunsaturated fatty acyl groups of phospholipids (35). Vitamin E levels and PUFA contents were shown to influence membrane susceptibility to lipid peroxidation, and below a certain vitamin E threshold, differences in membrane susceptibility to peroxidation could be reason ably predicted based on the content of individual peroxidizable fatty acids only (31). Furthermore, a strong positive correlation was found between heart vitamin E and n-3 fatty acids of total lipids in heart membranes of rats, which suggests that n-3 PUFA in cell membranes are efficiently

protected against lipid peroxidation (33). Individual data from this study showed that only after an induced peroxidative stress, i.e., a single MEKP injection, was there a positive correlation between vitamin E levels and PI values and a negative correlation between LPO levels and PI values in hearts from FO-fed rats (Fig. 2). No such correlation was found in hearts from TSCO-treated rats in either of the dietary groups.

The products of lipid peroxidation are mainly the hydroperoxides of fatty acyl moieties. In the present study, the lower levels of highly PUFA of heart PC induced by MEKP are not reflected in higher levels of LPO in heart. It is suggested that membrane-bound phospholipase A2 produces an "antioxidant" effect by excising LPOs from the membrane, thereby preventing them from participating in free radical propagation reactions (36). The membrane-bound phospholipase A2 is hence considered as one of the scavenger and antioxidant enzymes that act to preserve cellular structure and function.

GSH in liver is thought to be used as a cysteine reservoir for GSH synthesis in peripheral organs during periods of enhanced consumption or limited supply (37,38) and as a defense mechanism against oxidative stress (39). It has been shown that the reduction of GSH levels in liver as a consequence of oxidative stress was more pronounced in livers from fasted than from fed rats (40). A significant reduction in liver GSH levels was only observed in unfasted rats 24 h after an MEKP injection (Table 5). The reduced level of liver GSH was not reflected in an increased oxidized level of GSH (GSSG). The liver and heart GSSG levels were less than 1 nmol/mg protein in both dietary groups (data not shown).

In summary, the effect of MEKP treatment on GSH levels on the levels of PUFA of membrane phospholipids and on tissue weights differed between heart and liver. One should therefore be cautious in extrapolating results from one tissue to another. The present results also support the idea that in studies on lipid peroxidation and radical-scavenging defense mechanisms, attention must be paid to the biochemical changes that are associated with fasting (14,41). In the present study, no deleterious effect of increased susceptibility to lipid peroxidation due to the n-3 fatty acid-enriched dietary intake was seen in heart and liver of unfasted animals. The reduced levels of highly PUFA in heart PC induced by MEKP were not reflected in higher levels of LPO in heart from FO-fed rats. The fate of the peroxidized PUFA will require further study.

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